

RESEARCH PAPER

Effect of mineral sulphur availability on nitrogen and sulphur uptake and remobilization during the vegetative growth of *Brassica napus* L.

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Abstract

Because it has a high demand for sulphur (S), oilseed rape is particularly sensitive to S limitation. However, the physiological effects of S limitation remain unclear, especially during the rosette stage. For this reason a study was conducted to determine the effects of mineral S limitation on nitrogen (N) and S uptake and remobilization during vegetative growth of oilseed rape at both the whole-plant and leaf rank level for plants grown during 35 d with 300 µM $^{34}\text{SO}_4^{2-}$ (control plants; +S) or with 15 µM $^{34}\text{SO}_4^{2-}$ (S-limited plants; -S). The results highlight that S-limited plants showed no significant differences either in whole-plant and leaf biomass or in N uptake, when compared with control plants. However, total S and ^{34}S (i.e. deriving from S uptake) contents were greatly reduced for the whole plant and leaf after 35 d, and a greater redistribution of endogenous S from leaves to the benefit of roots was observed. The relative expression of tonoplast and plasmalemma sulphate transporters was also strongly induced in the roots. In conclusion, although S-limited plants had 20 times less mineral S than control plants, their development remained surprisingly unchanged. During S limitation, oilseed rape is able to recycle endogenous S compounds (mostly sulphate) from leaves to roots. However, this physiological adaptation may be effective only over a short time scale (i.e. vegetative growth).

Key words: ^{15}N labelling, ^{34}S labelling, sulphate, sulphur limitation, S transporter.

Introduction

Winter oilseed rape (*Brassica napus* L.) has become a plant of major agro-economic importance, with a yield of 47 millions tonnes worldwide in 2007 (FAO), and has a wide range of uses (oil production, animal feeding, alternative fuel, etc.). Moreover, winter oilseed rape is also considered to be an excellent rotation crop for cereals as it enhances suppression of soil-borne pathogens either by the release of biocidal compounds or by improvements in subsoil macro-porosity caused by its deep taprooting system (Kirkegaard *et al.*, 1997). As for other large cropping systems, its intensive culture requires important amounts of nitrogen (N), sulphur (S), phosphorus (P), and potassium (K) fertilizers. Amongst these fertilizers, N plays a major role. Even if oilseed rape is characterized by a high capacity for N uptake (Lainé *et al.*, 1993), which makes it suitable as

a catch crop species to limit N leaching in the aquifer during the autumn–winter season, it requires large amounts of N. Therefore, high N availability is strongly correlated with high yield and seed quality. For example, depending on site conditions, the optimum seed yield occurs in the range of 180 kg N ha⁻¹ to 220 kg N ha⁻¹ (Jackson, 2000). The main effects of increasing N status in oil seed rape have been shown to be an increase in leaf number and area (Gammelvind *et al.*, 1996; Leleu *et al.*, 2000; Sveenjak and Rengel, 2006), leaf chlorophyll content (Ogunlela *et al.*, 1989), and pod number and area (Gammelvind *et al.*, 1996; Hocking *et al.*, 1997; Leleu *et al.*, 2000).

S is also an essential element for plant growth because it is present in major metabolic compounds such as amino acids (methionine and cysteine), glutathione, proteins, and

sulpho-lipids. However, S availability has been decreasing in many areas of Europe during the last two decades (Schnug, 1991; McGrath *et al.*, 1996; Zhao *et al.*, 1999). Oilseed rape, as with most *Brassicaceae*, has greater S requirements than other large crop species such as wheat or maize. For example, the production of 1 tonne of rape seeds requires ~16 kg of S (McGrath and Zhao, 1996; Blake-Kalff *et al.*, 2001), compared with 2–3 kg for each tonne of grain in wheat (Zhao *et al.*, 1999).

Therefore, oilseed rape is particularly sensitive to S deficiency or limitation, which reduces both seed quality (Asare and Scarisbrick, 1995; De Pascale *et al.*, 2008) and yield by ~40% (Scherer, 2001). Such S deficiencies can be the result of a combination of processes. S-containing fertilizers such as superphosphate have been superseded by fertilizers containing little or no S (Zhao *et al.*, 1997, 1999), while a massive decrease of S inputs from atmospheric deposition has been recorded during the last three decades. Moreover, it can also be suggested that the S requirements of many crops have increased as a result of intensive agriculture and optimization during plant breeding programmes.

S requirement and metabolism in plants are closely related to N nutrition (Reuveny *et al.*, 1980), and N metabolism is also strongly affected by the S status of the plant (Janzen and Bettany, 1984; Duke and Reisenauer, 1986). A deficiency in S supply has been shown to depress the uptake of nitrate and the activity of nitrate reductase in maize and spinach (Friedrich and Schrader, 1978; Prosser *et al.*, 2001), and to result in transient or steady-state nitrate accumulation in maize, wheat, and oilseed rape (Dietz, 1989; McGrath and Zhao, 1996; Gilbert *et al.*, 1997). Fismes *et al.* (2000) have shown using field-grown oilseed rape that S deficiency can reduce nitrogen use efficiency (NUE: ratio of harvested N to N fertilization) and that N deficiency can also reduce sulphur use efficiency (SUE).

During the autumn–winter period, oilseed rape is in a vegetative stage, with the shoot present as a rosette of leaves (i.e. rosette stage). During this early development, leaves represent a major store of nutrients which can be remobilized from old to younger leaves or from senescing leaves to early reproductive tissues, as shown more specifically for N (Malagoli *et al.*, 2005b). Therefore, the leaves appearing during the rosette stage play a crucial role for seed filling and contribute to the maintenance of grain yield. For example, because of a reduced N uptake occurring after flowering (Malagoli *et al.*, 2004), nearly 75% of N content in reproductive tissues of oilseed rape is derived from N mobilization occurring mostly from leaves and stems (Malagoli *et al.*, 2005a). Noquet *et al.* (2004) reported that removal of 50% of the leaves present at the end of the rosette stage resulted in a 30% decrease in seed yield in oilseed rape. The initiation and dynamics of foliar senescence depend on leaf age but can also be modulated by different biotic or abiotic factors (Weaver *et al.*, 1998; Noh and Amasino 1999; He and Gan, 2002; Pourtau *et al.*, 2004). For instance, environmental factors such as mineral

N limitation (Gombert *et al.*, 2006; Kim *et al.*, 2007) or drought conditions (Thomas and Stoddart, 1980) may accelerate the initiation of leaf senescence and lead to many subcellular changes, including an increase in protease activities (Matile, 1982) which could be the result of disappearance of protease inhibitors in older leaves (Desclos *et al.*, 2008). In leaves, senescence may result in the mobilization of >70% of leaf proteins, with a preferential proteolysis of plastidial proteins such as ribulose-1,5-biphosphate carboxylase oxygenase (Rubisco) (Srivalli *et al.*, 2001). In *Brassica oleracea* L. (broccoli), 44% of the total proteolytic activities of senescent tissues were dependent on cysteine and serine proteases (Coupe *et al.*, 2003).

Developing leaves are the first ones to show symptoms of S deficiency (Blake-Kalff *et al.*, 1998). In the later stages of oilseed rape development, S deficiency can lead to slower growth and fewer leaves. Young leaves can become chlorotic and have reduced photosynthetic activity. Ahmad and Abdin (2000) demonstrated that high S fertilization increases Rubisco, chlorophyll, and protein contents in fully expanded upper leaves of *Brassica juncea* L. (mustard) and *Brassica campestris* L., which implies a better photosynthetic activity in comparison with plants grown without S. Rubisco contains 120 cysteines and 168 methionines per molecule (Miziorko and Lorimer, 1983). Therefore, Rubisco seems to be an obvious target for mobilization when S amino acid synthesis is restricted by S deficiency (Ahmad and Abdin, 2000). Whilst any decrease of Rubisco affects the photosynthesis rate, the decline of chlorophyll also contributes to the breakdown of photosynthesis when S is deficient. Chlorophyll degradation has been observed by Blake-Kalff *et al.* (1998) in oilseed rape, particularly in the youngest leaves of plants grown on nutrient solution containing no S and high N, but not in leaves of plants grown on no S and low N. They also observed that when sulphate is removed from the nutrient solution, the glutathione concentration decreased rapidly in the middle and youngest leaves. The uptake and subsequent distribution of sulphate to the leaves is closely regulated in response to demand (Blake-Kalff *et al.*, 1998). For instance, developing leaves are strong S sinks, but show a net loss of S after full expansion (Sunarpi and Anderson, 1996).

Because the appearance of S deficiency is fairly recent in European agriculture, research on crop S nutrition still lags far behind that on other major nutrients such as N. For instance, the way plants cope with reduced N availability through increased senescence of older leaves, which induce proteolysis leading to increased N remobilization to sink tissues, has been described in many plant species. However, the physiological effects of S limitation are less clear at the plant level. As a consequence, the objective of the present study was to examine the effects of mineral S limitation in oilseed rape on N and S uptake and remobilization during vegetative growth at both the whole-plant and leaf rank levels, using simultaneous ^{34}S and ^{15}N labelling, in order to determine how plants compensate for a reduced S availability.

Materials and methods

Hydroponic experiment

Seeds of oilseed rape (*B. napus* L. cv. Capitol) were sterilized by exposure to 80% ethanol for 30 s followed by 20% sodium hydrochlorite for 20 min. After several washes in demineralized water, seeds were placed on moist filter paper in plastic tanks under dark conditions for 2 d. Just after first leaf emergence, seedlings were transferred to hydroponic solution (18 seedlings per 12.0 l plastic tank) in a greenhouse with a thermoperiod of 20 °C (day) and 18 °C (night). Natural light was supplemented with phytor lamps [150 µmol m⁻² s⁻¹ of photosynthetically active radiation (PAR) at canopy height] for 16 h. The basic nutrient solution contained 0.40 mM KH₂PO₄, 0.15 mM K₂HPO₄, 2 mM KCl, 3.0 mM CaCl₂, 0.20 mM FeNaEDTA, 14 µM H₃BO₃, 5 µM MnSO₄·H₂O, 3 µM ZnSO₄·7H₂O, 0.7 µM CuSO₄·5H₂O, 0.7 µM (NH₄)₆Mo₇O₂₄, and 0.1 µM CoCl₂. This basic nutrient solution was renewed weekly and supplemented twice a week with KNO₃ and MgSO₄ to a concentration of 1 mM and 300 µM, respectively (Fig. 1), in order to be in optimal S conditions for growth for non-vernalized rosette plants. After 6 weeks, the plants were transferred to 4.0 l plastic pots (one plant per pot), and were then divided into two groups for the application of two contrasting levels of mineral S.

Application of S treatments and tissue sampling

Half the plants (control plants; +S) were supplied with 300 µM SO₄²⁻ and the remaining plants (S-limited plants; -S) were supplied with 20-fold less S (15 µM SO₄²⁻). Mineral S treatments were applied during 35 d with 1 mM KNO₃ (Fig. 1) and nutrient solution was renewed every week. Seven days before each harvest, plants were supplied with a labelled nutrient solution containing, ¹⁵NO₃ (2.5% atom excess) and ³⁴SO₄²⁻ (1% atom excess). Four plants (i.e. replicates) of both sets (control and S-limited plants) were harvested at day 0 and after 7, 14, 21, 28, and 35 d of treatment. The whole root system, stem, leaf blades (LBs), and leaf petioles were separated and weighed for determination of their fresh matter. Leaves were separated based on the date of their ontogenetic appearance (defined as the leaf rank number). The leaf rank number was ordered from the oldest to the youngest

leaves (i.e. from base to canopy). For each leaf, the leaf greenness content was measured using a SPAD-502 (Minolta, Tokyo, Japan) apparatus (Rossato *et al.*, 2001), and leaf area was determined with a LI-COR 300 area meter (LI-COR, Inc. Lincoln, NE, USA). Leaves as well as other plant tissues were frozen in liquid N₂ and stored at -80 °C until further isotope ratio mass spectrometry (IRMS), biochemical, and molecular analysis.

Preparation of ³⁴S

Elemental S enriched in ³⁴S (98% atom excess) was obtained from Trace Sciences International, France. A digestion procedure using 16.5 N HNO₃ was used to convert elemental S to sulphate (Zhao *et al.*, 1996). Briefly, 100 mg of elemental S was weighed into a Pyrex digestion tube. A 10 ml aliquot of HNO₃ was then added. Digestion was carried out in a programmable heating block with the temperature slowly rising up to 200 °C, and then kept at this temperature for 2 h. The tube was then cooled and the solution (called S1) was conserved. The remaining elemental S in the tube was washed with demineralized water and the washing solution was pooled with the previous solution (S1). These operations were repeated a second time. The different solutions were then recovered and K₂CO₃ was added.

The remaining solution was transferred to a 100 ml volumetric flask. Analysis of nitrate and sulphate concentrations in this stock solution was carried out using ion chromatography (Dionex DX100, CA, USA, with a conductivity detector). The eluent consisted of 1.8 mM Na₂CO₃ and 1.7 mM Na₂HCO₃, and was pumped isocratically over an AS17 guard column. The analysis of the stock solution showed a final recovery of ~82% of ³⁴SO₄²⁻, and both nitrate and sulphate concentrations were taken into account during plants N and S treatments.

Determination of total N and S content

An aliquot of each plant organ (roots, stems, LBs, and leaf petioles) was freeze-dried, weighed for dry matter (DW) determination, ground to a fine powder, and placed into tin capsules for isotopic analysis. The total N (¹⁴N, ¹⁵N) and S contents (³²S, ³⁴S) in plant samples were determined with a continuous flow isotope mass spectrometer (Isoprime, GV Instrument, Manchester, UK) linked to a C/N/S analyser (EA3000, Euro Vector, Milan, Italy):

Total N (N_{tot}) content in a tissue 'i' at a given time 't' was calculated as:

$$N_{tot,i,t} = \%N_{i,t} \times DW_{i,t} / 100$$

The natural ¹⁵N abundance (0.3663±0.0004%) of atmospheric N₂ was used as a reference for ¹⁵N analysis. Nitrogen derived from current N uptake (N_{upt}) in a given organ was calculated as follows:

$$N_{upt} = N_{tot} \times [E (\%) / E_S (\%)]$$

where E (%) is the atom % ¹⁵N excess in a given organ and E_S is the nutrient solution atom % ¹⁵N excess (2.5%).

As for N, total S (S_{tot}) content in a tissue 'i' at a given time 't' was calculated as:

$$S_{tot,i,t} = \%S_{i,t} \times DW_{i,t}$$

For ³⁴S enrichment, the natural isotope abundance of 4.255% was withdrawn to the value from sample ³⁴S abundance, and sulphur derived from current S uptake (S_{upt}) in a given organ was then calculated as follows:

$$S_{upt} = S_{tot} \times [E (\%) / E_S (\%)]$$

where E (%) is the atom % ³⁴S excess in a given organ and E_S is the nutrient solution atom % ³⁴S excess (1%).

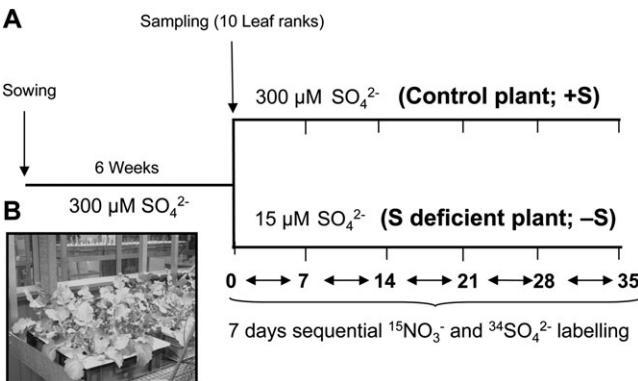


Fig. 1. (A) Culture conditions and experimental design used to provide two contrasting levels of S nutrition. Six weeks before the start of the experiment (i.e. day 0), the plants were grown on optimal S solution (300 µM SO₄²⁻). On day 0 and after subsequent sampling, half the plants were supplied with 300 µM SO₄²⁻ (control plant; +S) and half with 15 µM SO₄²⁻ (S-limited plants; -S) with sequential double labelling during the 7 d preceding each harvest. (B) The inset shows a picture of oilseed rape plants growing in aerated hydroponic solution in the greenhouse.

Determination of S mobilization and partitioning of S derived from uptake or mobilization within tissues

The mobilized S content (Smob) from or towards each tissue between two dates (i.e. day 35 to day 0) and presented in Fig. 4 was calculated by subtracting the accumulated S derived from uptake between these two dates (Supt) from the change in total S content (Stot_{t2}–Stot_{t1}) according to the following equation:

$$\text{Smob} (t_2 t_1) = (\text{Stot}_{t_2} \text{Stot}_{t_1}) \text{Supt}$$

Therefore, positive values of mobilized S represent S that is mobilized to this tissue, while negative values correspond to a net mobilization of S from this tissue. The addition of all values corresponding to mobilized S from tissues (i.e. exported) was then considered to represent 100% and the mobilization of S towards the sink tissues (i.e. imported) was calculated as a percentage of the total S mobilized (Fig. 4). The same calculation was carried out for total S taken up.

Determination of SO₄²⁻ content

Sulphate was measured by extracting 30 mg of freeze-dried plant material in 1.5 ml of 50% ethanol solution at 40 °C for 1 h. After centrifugation (20 min; 10 000 g) the supernatant (called S1) was recovered and 1.5 ml of 50% ethanol was added to the pellet. After a new incubation (1 h; 40 °C) and centrifugation (20 min; 10 000 g), the remaining supernatant was taken up and added to the previous supernatant (S1). All these operations (i.e. incubation and centrifugation) were repeated twice but now with 1.5 ml of ultra-pure water and incubation at 95 °C. All supernatants were finally pooled then air-dried for 16 h without heating. The dry residues containing both nitrate and sulphate were solubilized in 1 ml of ultra-pure water. Thereafter, nitrate and sulphate concentrations in the extracts were determined by using ion chromatography (Dionex DX100, with a conductivity detector). The eluent solution consisted of 1.8 mM Na₂CO₃ and 1.7 mM Na₂HCO₃, and was pumped isocratically over an AS17 guard column.

RNA extraction

Total RNA was extracted from 200 mg of LB fresh matter. Frozen samples were ground to a powder with a pestle in a mortar containing liquid nitrogen. The resulting powder was suspended in 750 µl of extraction buffer [0.1 M TRIS, 0.1 M LiCl, 0.01 M EDTA, 1% SDS (w/v), pH 8] and 750 µl of hot phenol (80 °C, pH 4). This mixture was vortexed for 30 s and, after addition of 750 µl of chloroform/isoamylalcohol (24:1), the homogenate was centrifuged at 15 000 g (5 min, 4 °C). The supernatant was transferred into 4 M LiCl solution (w/v) and incubated overnight at 4 °C. After centrifugation (15 000 g, 30 min, 4 °C), the pellet was suspended in 250 µl of sterile water. A 50 µl aliquot of 3 M sodium acetate (pH 5.6) and 1 ml of 96% ethanol were added to precipitate the total RNA for 1 h at -80 °C. After centrifugation (15 000 g, 20 min, 4 °C), the pellet was washed with 1 ml of 70% ethanol, then centrifuged at 15 000 g for 5 min at 4 °C. The resulting pellet was dried for 5 min at room temperature and resuspended in sterile water containing 0.1% SDS and 20 mM EDTA. Quantification of total RNA was performed by spectrophotometry at 260 nm (BioPhotometer, Eppendorf, Le Pecq, France) before reverse transcription and quantitative PCR (Q-PCR) analyses.

Reverse transcription and Q-PCR analysis of BnSultr genes

For reverse transcription, 1 µg of total RNA was converted to cDNA with an 'iScript cDNA synthesis kit' according to the manufacturer's protocol (Bio-Rad, Marne-la-Coquette, France). For Q-PCR amplification, primers BnSultr1 and BnSultr4 encoding vacuolar sulphate transporters in *B. napus* L. were designed from the BnSultr1;1 sequence (accession no: AJ416460), the BnSultr1;2

sequence (accession no: AJ311388), the BnSultr4;1 sequence (accession no: AJ416461), and the BnSultr4;2 sequence (accession no: AJ555124) previously published by Parmar *et al.* (2007). Q-PCR amplifications were performed by using BnSultr1;1 forward primer 5'-AGATATTGCGATCGGACCAAG-3' and reverse primer 5'-GAAAACGCCAGCAAAGAAAG-3'; BnSultr1;2 forward primer 5'-GGTGTAGTCGCTGGAATGGT-3' and reverse primer 5'-AACGGAGTGAGGAAGAGCAA-3'; BnSultr4;1 forward primer 5'-GACCAGACCCGTTAAGGTCA-3' and reverse primer 5'-TTGGAATCCATGTGAAGCAA-3'; and BnSultr4;2 forward primer 5'-AGCAAGATCAGGGATTGTGG-3' and reverse primer 5'-TGCAACATTGTGGGTGT_{Ct}-3'. The EF1- α gene (accession no: DQ312264) was used as an internal control gene and was amplified using the primers described above. Q-PCRs were performed with 4 µl of 100× diluted cDNA, 500 nM of primers, and 1× SYBR Green PCR Master Mix (Bio-Rad) in a ChromoFour System (Bio-Rad). For each pair of primers, a threshold value and PCR efficiency have been determined using a cDNA preparation diluted >10-fold. For both pairs of primers, PCR efficiency was ~100%. The specificity of PCR amplification was examined by monitoring the presence of the single peak in the melting curves after Q-PCRs and by sequencing the Q-PCR product to confirm that the correct amplicons were produced from each pair of primers (Biofidal). In addition, Blastn analysis (www.ncbi.nlm.nih.gov/blast/Blast.cgi) was performed in order to check the correct amplification of the target cDNA, for the four different sulphate transporters (BnSultr1 and BnSultr4 families). For each sample, the subsequent Q-PCRs were performed in triplicate and the relative expression of the four different sulphate transporters in each sample was compared with the control sample [corresponding to control plants (+S) at day 0] and was determined with the $\Delta\Delta Ct$ method using the following equation (Livak and Schmittgen, 2001):

$$\text{Relative expression} = 2^{-[\Delta Ct_{\text{sample}} - \Delta Ct_{\text{control at day 0}}]},$$

$$\text{with } \Delta Ct = Ct_{\text{BnSultr1.1, 1.2, 4.1, or 4.2}} - Ct_{\text{EF1-}\alpha}$$

where Ct refers to the threshold cycle determined for each gene in the exponential phase of PCR amplification. Using this analysis method, relative expression of the different sulphate transporter genes in the control sample at day 0 of the experiment was equal to 1 (Livak and Schmittgen, 2001), and the relative expression of other treatments was then compared with the control at day 0, on this basis.

Statistical analysis

Results are presented as mean values ±SE for four replicates ($n=4$). The effects of mineral S were determined by analysis of variance (ANOVA), and according to a comparison of the means (Tukey *t*-test), with MINITAB13 on Windows (Minitab Inc., State College, PA, USA). When the normality law of data was not respected, the non-parametric test of Kruskal-Wallis was used. Statistical significance was postulated for $P < 0.05$, and different letters in the figures indicate that mean values are significantly different at a given date between treatments.

Results

Plant biomass analysis

Figure 2A shows the influence of S availability on the growth of whole plant, whole LB, and root for control plants (+S) and S-limited plants (-S). The dynamic of biomass production remained similar in +S and -S plants for whole plant, whole LB, or roots, where progressive

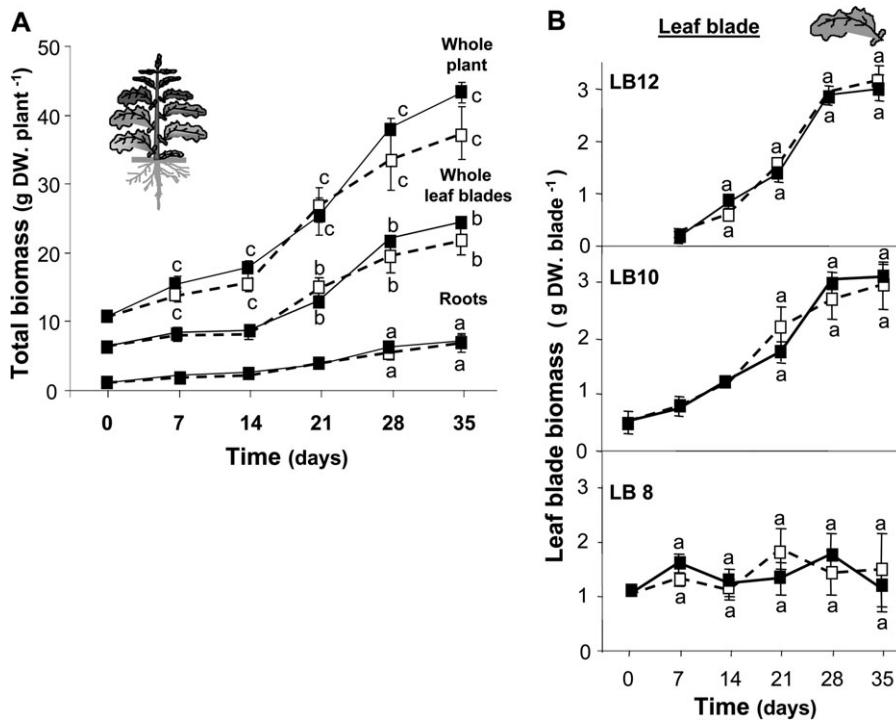


Fig. 2. Changes of (A) whole plant, whole leaf blade (LB), and root biomass, and (B) LB biomass of leaf ranks 8, 10, and 12 of oilseed rape for control plants (+S; filled symbols) and S-limited plants (-S; open symbols) during the 35 d of treatment. Vertical bars indicate \pm SE ($n=4$) when larger than the symbol. Different letters indicate that mean values are significantly different at a given date between treatments ($P < 0.05$).

increases were observed for both treatments. For example, at the end of the experiment (i.e. day 35), whole-plant biomass reached 39 ± 3 g DW plant⁻¹ for +S and -S plants. At this date, LB and root biomass represented ~60% and 15%, respectively, of whole-plant biomass.

LB biomass of three different leaf ranks, corresponding to old, mature, and young leaves (i.e. LB8, 10, and 12, respectively), was also monitored in response to S availability (Fig. 2B). For control plants (+S), LB8 biomass increased from day 0 to day 7 and then remained relatively constant until day 35. For both LB10 and 12, biomass progressively increased until the end of the experiment. No significant difference ($P > 0.072$) was observed in LB biomass production between mineral S treatments (i.e. +S or -S).

N, S, and accumulated ^{15}N and ^{34}S contents

Whole-plant N and ^{15}N contents (i.e. derived from uptake) increased at a constant rate during the experiment and were not affected by S nutrition (Fig. 3A). Whole-plant N content reached 440 ± 10 mg plant⁻¹ at the end of the experiment for both treatments, with ~50% derived from N uptake (^{15}N).

Whole-plant S and accumulated ^{34}S contents (i.e. derived from uptake) are presented in Fig. 3B. For +S plants, whole-plant S and ^{34}S contents greatly increased during the experiment. At day 35, S derived from uptake represented ~84% of total S. For -S plants, whole-plant S and ^{34}S contents remained relatively constant during the overall

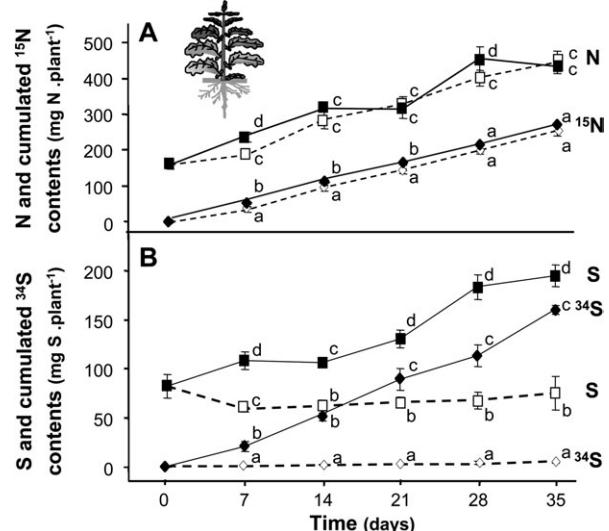


Fig. 3. Changes of (A) whole-plant N and ^{15}N (i.e. deriving from uptake) and (B) whole-plant S and ^{34}S (i.e. deriving from uptake) of oilseed rape for control plants (+S; filled symbols) and S-limited plants (-S; open symbols) during the 35 d of treatments. Vertical bars indicate \pm SE ($n=4$) when larger than the symbol. Different letters indicate that mean values are significantly different at a given date between treatments ($P < 0.05$).

experiment. Due to the reduced S availability, not more than 6 ± 2 mg of S were taken up throughout the experiment. As a consequence, the S deriving from uptake was <5% of that taken up by control plants.

S partitioning and remobilization

Figure 4 represents the partitioning of S taken up (determined on the basis of ^{34}S enrichment, see Materials and methods for details) and remobilized S (estimated from the unlabelled endogenous S present on day 0) through the main plant tissues (leaves, petioles, stems, and roots) between day 0 and 35. For +S plants (Fig. 4A), the S taken up was mostly allocated to the leaves (55%) and to the roots (27%). The limitation of S availability greatly reduced total S uptake (~95%) and changed the ^{34}S partitioning within the different plant tissues (Fig. 4B). For -S plants, ~65% of S taken up was found in the roots, whereas <23% was found in leaves. The remobilization of endogenous S was also studied. For +S plants (Fig. 4A), 26.8 mg of S were remobilized from leaves between day 0 and day 35, and exported mainly to the stem (79%), and to a lesser extent to

the roots (13%). The amount of S remobilized from -S plants was the same as for +S plants ($P > 0.062$), and leaves represented the major source organ (88%) while petioles contributed 12% (Fig. 4B). However, reduced S availability strongly modified the partitioning of endogenous S and, unlike for control plants, most remobilized S was supplied to the roots (60%), which, as with ^{34}S uptake, appeared as the main S sink. When compared with control plants, five times more of the remobilized S was partitioned to the roots in -S plants. Despite the roots being such a strong sink in -S plants, quantitatively they had 2-fold less total S than +S plants while having the same dry matter, as seen previously (Fig. 2A).

S and accumulated ^{34}S dynamics of LB and roots:

LB and root S contents were also studied on a kinetic basis in response to S availability (Fig. 5A). For +S plants, the S content of LB8 rapidly increased to 6.6 ± 0.9 mg at day 7 and then decreased to <4 mg S tissue $^{-1}$ thereafter. For LB10, the S content remained stable from day 0 until day 14 ($P < 0.023$) then increased by ~44% at day 35. For LB12, the S content was increased by ~28% at day 14 and then continued to increase until the end of the experiment (Fig. 5A).

For -S plants, the S contents of LB8 and 10 were significantly affected after 7 d of treatment (Fig. 5A). The S content rapidly decreased by 4.2 ± 1 mg and 2.73 ± 0.3 mg from day 0 to 7, respectively, then it slowly decreased until day 21 and remained stable thereafter. For LB12, which appeared during the first week after application of S limitation (i.e. at day 7), S content remained stable throughout the experiment ($P < 0.046$; Fig. 5A).

The S content in the roots of +S plants increased steadily throughout 35 d. More surprisingly, but to a lesser extent, root S content also increased throughout 35 d in -S plants despite a strong S limitation (Fig. 5A).

Analysis of accumulated ^{34}S content in plant tissues (Fig. 5B) showed a steady increase in all plant tissues (roots, and LB8, 10, and 12) of +S plants, while it remained at a very low level in -S plants.

Remobilization of endogenous S content of leaf blade and roots

The remobilization of endogenous S from or to different plant tissues is shown in Fig. 5C. For older leaves (LB8 and 10), it clearly showed that they act as source tissues, exporting S, whatever the S supply, and with similar contributions. However, S limitation slightly increased ^{32}S remobilization during the first 7 d. In the meantime, younger leaves (LB12) and roots clearly acted as sink tissues throughout the experiment as their ^{32}S content increased whatever the S supply. However, S limitation decreased the remobilization of S to younger leaves, while greatly increasing it to the roots, which became the main sink tissue. The overall results showed that during S limitation, S remobilization was maintained from older

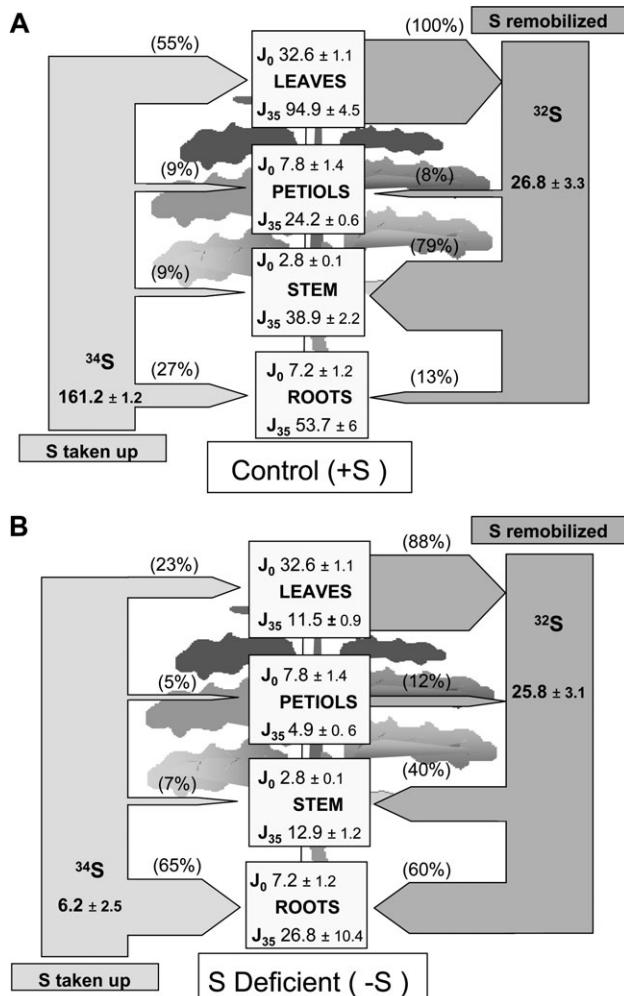


Fig. 4. Partitioning (in %) of total S taken up (^{34}S) and total remobilized S (^{32}S , present on day 0) in leaves, petioles, stem, and roots of oilseed rape for control plants (+S; A) and S-limited plants (-S; B) between day 0 and day 35. Values are given as the mean $\pm \text{SE}$ ($n=4$). The thickness of the arrows represents the relative importance (in % of the total S taken up or the total mobilized S) of each flux for accumulated total S taken up or total remobilized S (for details, see Materials and methods).

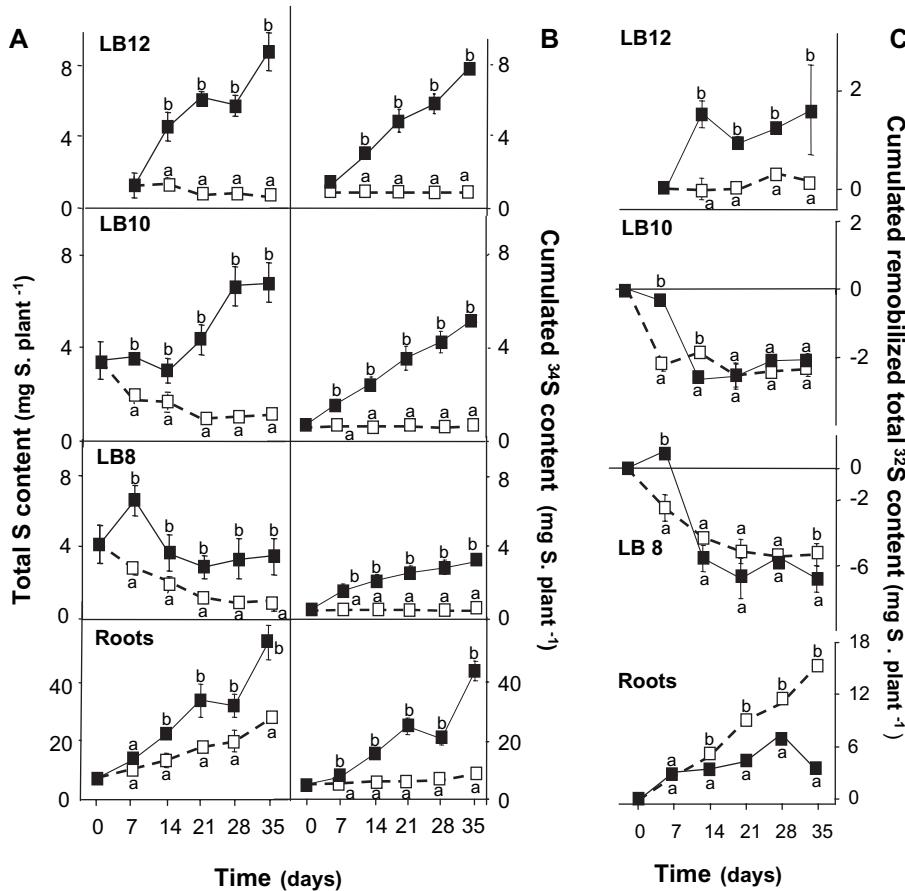


Fig. 5. Evolution of (A) total S, (B) accumulated ^{34}S taken up, and (C) accumulated remobilized ^{32}S contents in leaf blade (LB) of leaf ranks 8, 10, 12 and roots of oilseed rape for control plants (+S; filled symbols) and S-limited plants (-S; open symbols) during 35 d of treatments. Vertical bars indicate $\pm\text{SE}$ ($n=4$) when larger than the symbol. Different letters indicate that mean values are significantly different at a given date between treatments ($P<0.05$).

leaves, but it was mostly used by roots instead of being used by young leaves. Nevertheless, the small amount of endogenous S allocated to LB12 was sufficient and crucial to maintain its growth rate (Fig. 2B), especially during its early development (i.e. leaf expansion) at the beginning of the experiment.

N/S ratio, N organic/S organic ratio, and sulphate contents:

The effect of S availability on the N/S ratio is presented in Fig. 6A. The N/S ratio in +S plants reached ~5 in leaves and ~4 in roots, then decreased with time for both tissues. When submitted to S limitation, the N/S ratio increased significantly from day 7 in the older leaves (LB8) and (LB10). However, in roots, this ratio was maintained in a much narrower range, reaching ~6 after 35 d of S limitation. When just organic compounds were considered (i.e. the difference between total N or S and mineral N or S, such as nitrate and sulphate, respectively; Fig. 6B), the N organic/S organic ratio fluctuated very slightly in roots whatever the S supply, while it was 2- to 3-fold increased in leaves of plants with large S supply, and was slightly decreased with S limitation ($P<0.038$).

The difference in the change of these ratio (N/S and N organic/S organic) can be fully explained by the fact that nitrate represents a very small proportion of total N, while sulphate was the main form of S, accounting for >86% of total S in these tissues. Therefore, S-sulphate contents usually increased in all plant tissues according to their growth rate under sufficient S nutrition, except for older leaves (LB8) for which sulphate contents decreased with time (Fig. 6C). Moreover, no sulphate was detected after 3 weeks of S limitation in LBs, while ~2 mg of S-sulphate were retained in roots, even after 35 d of treatment. If sulphate concentration is expressed in mg S-sulphate g^{-1} DW, it appears that during early LB development there is an accumulation of sulphate followed by a progressive decline of sulphate content, along with senescence processes. S limitation reduced this content to very low values after 3 weeks in LBs, while in roots a steady sulphate content of ~0.5 mg $\text{S-SO}_4^{2-} \text{ g}^{-1}$ DW was achieved after 14 d.

Relative expression of sulphate transporters

Figure 7 presents the relative expression of plasmalemma (*BnSultr 1;1* and *BnSultr 1;2*) and tonoplast (*BnSultr 4;1*

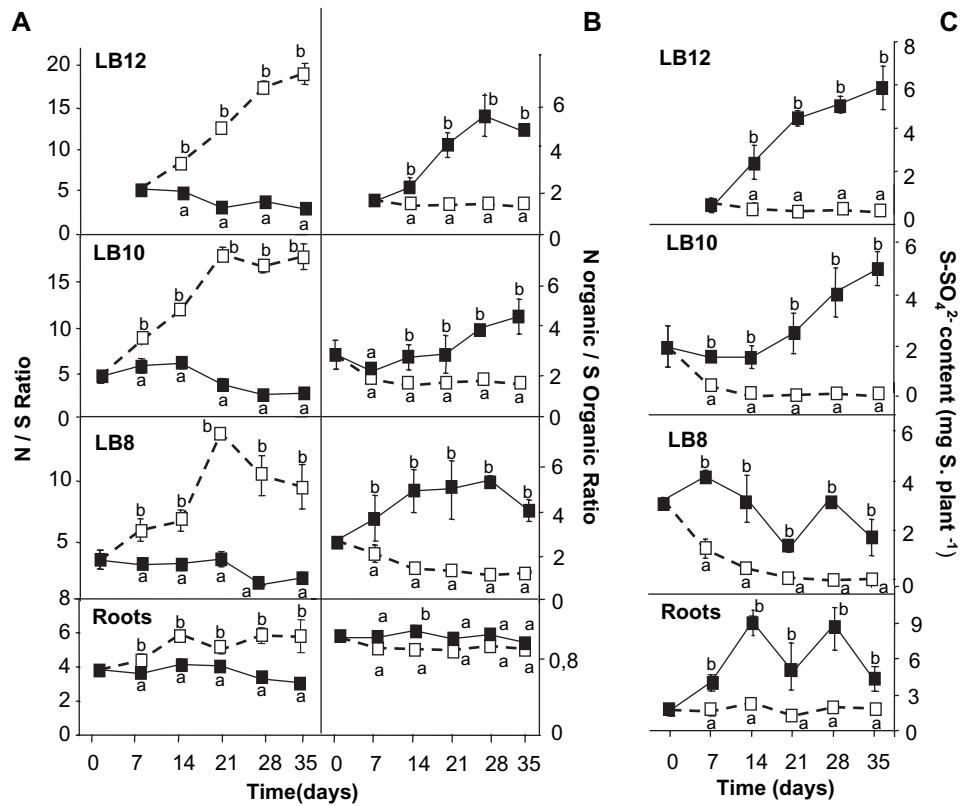


Fig. 6. Changes in (A) N/S ratio, (B) N organic/S organic ratio, and (C) S-SO₄²⁻ content in leaf blade (LB) of leaf ranks 8, 10, 12 and roots for control plants (+S; filled symbols) and S-limited plants (-S; open symbols) during 35 d of treatments. Organic N (or S) is represented by the difference between total N (or S) and nitrate (or sulphate). Vertical bars indicate ±SE ($n=4$) when larger than the symbol. Different letters indicate that mean values are significantly different at a given date between treatments ($P < 0.05$).

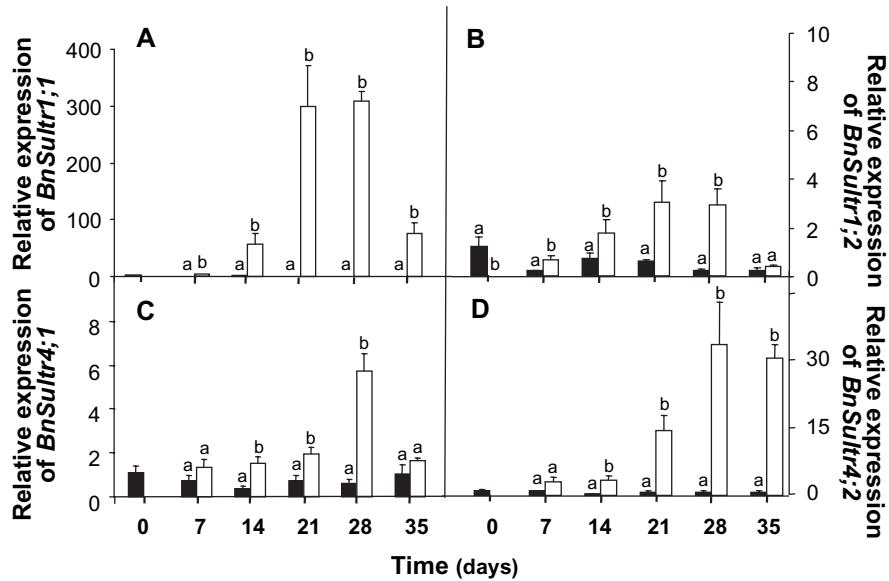


Fig. 7. Relative expression of root plasmalemma (A) BnSultr1.1 and (B) BnSultr1.2, and tonoplast (C) Bnsultr4.1 and (D) Bnsultr4.2 in oilseed rape for control plants (+S; dark bars) and S-limited plants (-S; open bars) during 35 d of treatment. Relative expression for each gene is the average ±SE ($n=4$) of Q-PCR analysis and is expressed relative to the day 0 initial control expression level for that gene. Different letters indicate that mean values are significantly different at a given date between treatments ($P < 0.05$).

and *BnSultr 4;2*) sulphate transporters in the roots of +S and -S plants. *BnSultr 1;1* was strongly induced in the -S treatment, reaching values of 300 before declining at 35 d. *BnSultr 4;2* was also strongly induced until 35 d. The other two transporters were induced to a lesser extent.

Discussion

Under field conditions, the availability of mineral S for plant growth and development would be mainly dependent upon soil availability, autumn/winter rainfall patterns, and atmospheric depositions of SO₂ and H₂S. However, S availability has been decreasing in many areas of Europe during the last three decades (Schnug, 1991; McGrath *et al.*, 1996; Zhao *et al.*, 1999). Oilseed rape, as with most *Brassicaceae*, has greater S requirements than other large crop species such as cereals. Therefore, the main objective of the present study was to examine the influence of S limitation on plant biomass and on the processes of S uptake, distribution, and remobilization during vegetative growth of oilseed rape at the rosette stage. In addition, the uptake and allocation of ³⁴S (i.e. deriving from recent ³⁴S uptake) and the remobilization of endogenous S (i.e. deriving from initial ³²S reserves, present at day 0) were studied in old and maturing leaves, which are considered to export large amounts of S (Parmar *et al.*, 2007) to growing tissues.

According to Hawkesford and De Kok (2006), in response to a limitation of S availability, the hypothetical initial responses involve optimization of S uptake and utilization of sulphate, accompanied by an increase in remobilization of inorganic S reserves from vegetative tissues and subsequent redistribution towards growing tissues. In the case of transient mineral S limitation perceived at the rosette stage, the present study revealed that sulphate limitation (15 µM versus 300 µM SO₄²⁻) applied for 35 d had no significant effect on whole-plant, whole LB, or root biomass production (Fig. 2A). These results are in agreement with field studies conducted by Zhao *et al.* (1993) where it was shown that there were no significant differences in dry matter accumulation for two different genotypes (Bienvenu and double low variety Cobra) grown with 0 or 100 kg S ha⁻¹. Moreover, in *B. oleracea*, Koralewska *et al.* (2007) reported that biomass allocation is not affected by low concentrations of sulphate in the root environment but only by the complete absence of S. It is generally considered that S availability may influence the NUE of oilseed rape, and vice versa (Schnug *et al.*, 1993; Fismes *et al.*, 2000), indicating that mineral S and N availabilities interact to affect S and N management by the plant (Janzen and Bettany, 1984; Kopriva and Rennenberg, 2004). At the rosette stage of oilseed rape, however, the present study also revealed that sulphate limitation had no significant effect on plant total N content or on N uptake (Fig. 3A). However, as expected, reduced S availability curtailed S accumulation and uptake in comparison with control plants, which continued to accumulate S

as normal. The sulphate limitation treatment started after a period of 51 d, during which plants were supplied with optimal levels of sulphate. This period of pre-culture almost certainly resulted in plants with a high initial S status, according to the high initial S-sulphate contents within the leaves which represents the main S source at the plant level at day 0 (and with up to 86% of S as sulphate; data not shown). Therefore, it may be hypothesized that the remobilization of endogenous S compounds was sufficiently efficient to maintain the growth of S-limited plants at a similar level to the control plants. However, oilseed rape samples collected in field conditions and grown with the recommended level of S fertilization (60–80 kg S ha⁻¹) also revealed quite a high sulphur content in leaves (up to 0.97% DW, by comparison with leaves of the present experiment with 1.03% DW), of which 75% was in a sulphate form (data not shown). These data suggest that plants used for the present experiment (86% of foliar S as sulphate) were very close in terms of S status to plants that are grown in field conditions under a conventional fertilization regime (63–76% of S as sulphate). They also suggest that oilseed rape is able to compensate for an S limitation over a short time scale (i.e. in comparison with its whole development cycle) through the fine management of N and S metabolism. This particular behaviour of oilseed rape to a limitation of S availability should be particularly relevant for the *Brassicaceae* (i.e. known to be sensitive to S limitation), and will be more clearly assessed in further studies.

For +S plants (Fig. 4A), almost 27 mg of ³²S were remobilized between day 0 and day 35. Leaves represented the sole export tissue, while the main sink tissues were stem (79%) and root (13%). For -S plants, leaves also represented the major source organ (Fig. 4B). However, petioles also contributed a little to the supply of mobilized S to sink tissues. The reduction of S availability also strongly modified the partitioning of mobilized S and, unlike for control plants, most of the remobilized S was supplied to the root (60%). When compared with control plants, five times more remobilized S was distributed to the root in -S plants. Despite this, quantitatively, roots of -S plants had 2-fold less total S than roots of +S plants. When remobilization fluxes (Fig. 4, 26.8 mg S plant⁻¹ and 25.8 mg S plant⁻¹) are compared with the reduction of S-SO₄²⁻ content after 35 d in -S plants (from 19.97±3.07 mg S-SO₄²⁻ plant⁻¹ at day 0 to 7.19±0.71 mg S-SO₄²⁻ plant⁻¹ at day 35), it appears that sulphate alone may account for ~59% of total S remobilization, the rest involving organic S.

According to Kopriva and Rennenberg (2004), O-acetyl serine (OAS) may be required for the transduction of the signal involved in the increase in expression of transporters and enzymes involved in SO₄²⁻ uptake and assimilation. Nevertheless, the increase of OAS can be blocked when N is limiting (Kim *et al.*, 1999). Thus, in limiting conditions of S fertilization, OAS may act as a signal of insufficient sulphide production and would act as a positive control on the expression of genes which encode enzymes of sulphate assimilation such as adenosine 5'-phosphosulphate reductase (APR) and on the capacities for SO₄²⁻ uptake

(Rouached *et al.*, 2008). The present results are in agreement with these findings because in conditions of S limitation there was a higher expression of both *BnSultr4;1* and *BnSultr4;2* (high affinity sulphate transporters suspected to be implicated in sulphate uptake by roots), as Maruyama-Nakashita *et al.* (2004) also reported.

These results also suggest that redistribution of S within the plant took place in response to limited S availability (Fig. 4). For +S plants (Fig. 4A), the S taken up was mostly allocated to the leaves (55%) and to the roots (27%). The limitation of S availability greatly reduced total S uptake and changed the ^{34}S partitioning within the different plant tissues (Fig. 4B). For -S plants, 65% of S taken up was thus found in the roots, while only 23% was found in leaves, with most of the latter distributed to young leaves (data not shown). Moreover, the total S content of LB8 and LB10 strongly decreased (Fig. 5), indicating a large remobilization of S compounds from the soluble fraction, principally as sulphate (Fig. 6C), which was reported to be mainly stored within the vacuole (Smith and Lang, 1988; Bell *et al.*, 1990, 1995; Cram, 1990; Sunarpi and Anderson, 1996, 1997; Eriksen *et al.*, 2001). In a parallel study, performed with the same genotype grown under the same conditions, Dubousset *et al.* (2009) reported that vacuolar sulphate is specifically remobilized from mature leaves and that this mobilization is related to an up-regulation of *BnSultr4;1* and/or *BnSultr4;2* gene expression. These authors also indicated that the relationship between sulphate mobilization and up-regulation of expression of *BnSultr4* genes is specifically dependant on the N availability. Moreover, this redistribution of S compounds to young developing leaves and roots was without any acceleration of leaf senescence processes (Dubousset *et al.* 2009). The authors hypothesized that this would maintain photosynthetic capacities of shoot tissues and subsequent metabolic activities within the whole plant (i.e. including uptake processes in the root).

In conclusion, the present study provides evidence that in the case of a transient mineral S limitation perceived at the rosette stage, oilseed rape, which is considered to be a high S-requiring plant, is able to maintain its growth by an optimization of N uptake and by the recycling of endogenous foliar S compounds (particularly SO_4^{2-}) predominantly from mature leaves (LB8 and 10). The results also demonstrate that under S limitation, the main ecophysiological strategy of oilseed rape is to maintain its root growth rate not by increasing S mobilization from leaves, but rather by re-orientating more of the S fluxes (uptake and remobilization) to the roots. However, it is not known whether S is transported as an inorganic form as sulphate. In the latter case, the study of the phloemic sulphate transporter (*BnSultr 1.3*) could be relevant, but it has not been possible yet to clone this gene in oilseed rape. Overall, plants under S limitation appear to optimize soil S capture by maintaining plant growth through targeted S remobilization to the roots and by increasing the expression of root S transporters.

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